

## ORIGINAL PAPER

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## DNA aberrations in urinary bladder cancer detected by flow cytometry and FISH

**Abstract** Detection of molecular alterations is of potential significance for diagnosis and prognosis in bladder cancer. Fluorescence in situ hybridization (FISH) allows visualization and quantitation of genes and chromosomes on a cell by cell level and can easily be applied to urinary cells. To evaluate the sensitivity of FISH for detection of DNA aberrations in bladder cancer, formalin-fixed tissues of 293 tumors were examined by FISH and flow cytometry (FCM). Centromere probes for the chromosomes X, Y, 1, 7, 9, and 17 were used for FISH analysis. FISH was more sensitive for detection of quantitative DNA aberrations than FCM. An aberration of at least one chromosome was found in 107 of 108 tumors (99%), which were tetraploid, aneuploid, or multiploid, and in 29 of 49 tumors (59%), which were diploid, by FCM. The frequency of FISH aberrations showed greater differences between pTa (47%) and pT1 tumors (85%;  $P < 0.0001$ ) than between stages pT1 and pT2–4 (98%). The marked genetic difference between pTa and pT1 tumors argues against the concept of grouping pTa and pT1 tumors together as “superficial bladder cancer.” The frequency of tumors with chromosomal aberrations detected by FISH increased with the number of chromosomes examined. Aneusomy was seen in 68% of grade 1 tumors examined for  $\geq 4$  chromosomes, suggesting that the cytological diagnosis of bladder cancer recurrences could be substantially improved by FISH.

**Key words** Bladder neoplasms · Flow cytometry · Fluorescence in situ hybridization · Chromosome Y · Chromosome X · Chromosome 1 · Chromosome 7 · Chromosome 9 · Chromosome 17 · Aneuploidy

### Introduction

Quantitative aberrations of the cellular DNA content occur frequently in bladder cancer [23]. Despite evidence for a diagnostic significance of DNA aneuploidy [5, 6, 24, 26], DNA measurement has not become a routine examination in bladder cancer patients. This is probably due to the need for expensive equipment and specially trained personnel to perform either flow cytometry (FCM) or DNA image cytometry.

Fluorescence in situ hybridization (FISH) is an alternative method for detection of a disturbed cellular DNA content. FISH allows visualization and therefore quantitation of chromosomes and genes on a cell by cell basis [9, 10]. FISH is technically easy and there is no need for specialized equipment. A major advantage of FISH as compared to total DNA measurement by FCM or image analysis is the capability to specifically examine chromosomes of interest for numerical aberrations. Since gains of chromosomes (polysomy) are frequently the result of a generalized alteration of the cellular DNA content involving most if not all chromosomes (tetraploidization), quantitation of a few chromosomes might be sufficient to detect DNA aneuploidy by FISH. Several studies have suggested that the sensitivity of FISH for detection of quantitative DNA aberrations exceeds the sensitivity of FCM [8, 9].

The aim of this study was to compare the sensitivity of FISH and FCM for detection of a disturbed DNA content in bladder cancer cells. In addition, the relationship between DNA aberrations and tumor grade and stage was examined.

### Materials and methods

#### Biopsy material

Formalin-fixed paraffin-embedded tissue samples of primary transitional cell carcinomas of the urinary bladder were available from the archives of the Institute for Pathology, University of Basel, Switzerland.

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land. A total of 293 tumors were selected for this study including 122 tumors that were previously examined by FISH for multiple chromosomal aberrations [18, 19, 21]. Tumor stage and grade were defined according to UICC [25] and WHO [14], using grades 1–3. Pure papillomas (grade 0) were excluded. Because of the limitations of transurethral biopsies in accurately determining the depth of invasion of higher stage bladder cancer, all tumors showing muscle invasion were categorized into one group (pT2–4). Ninety-six tumors were confined to the bladder mucosa (pTa), 67 showed minimal invasion limited to the lamina propria (pT1), and 130 were muscle-invasive (pT2–T4). Forty-five tumors were grade 1, 109 were grade 2, and 139 were grade 3. Fifty-micrometer sections were taken from all tissue blocks. Nuclei were enzymatically dissociated as previously described [19].

#### Flow cytometry

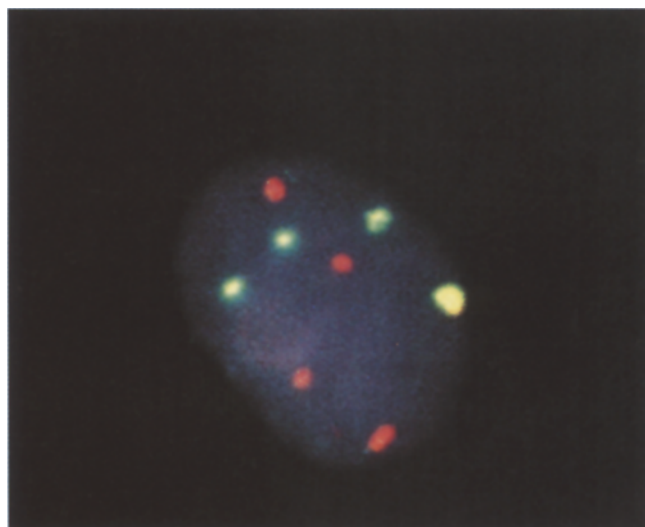
FCM for DNA measurement was performed in 237 cases. Nuclei were stained with propidium iodide. Dissociated nuclei were analyzed with a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA). For each specimen 10 000–20 000 events were collected. The Multicycle Program (Phoenix Flow Systems, San Diego, CA) was used for data analysis. The lowest G0/G1 peak was considered diploid and given a DNA index (DI) of 1.00. The DI of other G0/G1 peaks was calculated as the ratio of their G0/G1 peak channel number of the diploid G0/G1 peak channel number of the histogram. Tumors with a nondiploid G0/G1 peak with a  $DI \leq 1.2$  were considered peridiploid. Tumors were defined as diploid if only one G0/G1 peak was present and the G2M peak was less than 15%. A tumor was categorized as tetraploid if the DI was 1.9–2.1 or if the G2/M peak of an otherwise diploid tumor was higher than or equal to 15%. Tumors with G0/G1 peaks not in the peridiploid ( $DI \leq 1.2$ ) or tetraploid (DI 1.9–2.1) range were considered aneuploid. Multiploid tumors were defined as having more than one nondiploid G0/G1 peak. The coefficient of variation (CV) was calculated for each G0/G1 peak. Only tumors with a diploid  $CV \leq 8\%$  were included in this study.

#### Fluorescence in situ hybridization

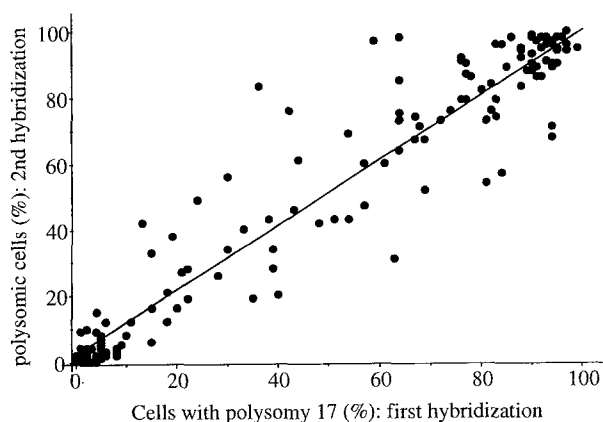
FISH was performed as previously described [19, 21, 22]. The following centromere probes were used: pY (chr. Y), pBAMX (chr. X), pUC177 (chr. 1), p7alphaTET (chr. 7), pHUR98 (chr. 9), and p17H8 (chr. 17). All probes were generously supplied by the UCSF Resource for Molecular genetics (Dr. Joe W. Gray, Director). Probe labeling was performed with biotin-14-dATP or digoxigenin-11-dUTP by nick translation using standard protocols. For all hybridizations a dual-labeling procedure using centromere probes together with other centromeric or locus-specific cosmid probes was used as described [18–22]. Cells on slides were denatured in 70% formamide/2X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate), pH 7, at 75 °C for 2.5 min. Pretreatment of slides was with proteinase K (2.0 µg/ml; Sigma) in PBS (pH 7.0) for 7 min at 37 °C. The hybridization mixture was denatured for 5 min at 75 °C. For centromere/cosmid dual labeling, 10 µl hybridization mixture consisted of 10 ng cosmid probe, 10–30 ng centromeric probe as well as 10 ng unlabeled, sonicated (200–500 basepairs) human placental DNA (Sigma) in 50% formamide, 10% dextran sulfate, and 2X SSC (pH 7). For dual hybridizations using two centromere probes, the hybridization mixture was composed of 10–30 ng centromeric probe and 10 ng unlabeled, sonicated (200–500 basepairs), herring sperm DNA (Sigma) in 55% formamide, 10% dextran sulfate, and 2X SSC (pH 7). Hybridization was performed overnight at 37 °C. Lymphocyte metaphase spreads were used as controls to assure probe specificity. The slides were washed and immunostained in five steps as previously described [19]. If hybridization signals were weak, presumably due to low hybridization efficiency, hybridization was repeated using the same protocol with an increased proteinase K concentration (up to 10 µg/ml). Proteinase K concentration was reduced if excessive nuclear damage was observed.

#### Scoring of FISH signals

Interpretation of centromere signals was as described [21, 22]. In brief, only cells with at least one bright signal for one of the applied probes were scored to avoid false chromosomal losses due to insufficient hybridization efficiency. Slides were only scored if at least two-thirds of cells were interpretable. Small lymphocytes and granulocytes were disregarded. At least 100 nuclei were analyzed. Tumors were stratified according to fraction of polysomic cells (cells with >two centromere signals) and according to their predominant population [19, 22]. A tumor was defined as polysomic for a chromosome if the fraction of polysomic cells was  $\geq 10\%$ . A loss of the Y chromosome was defined as the presence of  $\geq 20\%$  of nullisomic cells. The predominant population of a tumor was defined as the largest abnormal (aneusomic) population having  $>20\%$  of total cells [20]. A loss of chromosome 9 was defined if a tumor was either monosomic for chromosome 9 ( $\geq 20\%$  monosomic cells) or its chromosome 9 copy number category (predominant population) was below the copy number category for all other chromosomes examined (except chromosome Y).



**Fig. 1** Dual labeling FISH with probes for centromere 9 (red) and centromere 1 (green). Dissociated cell from a formalin-fixed bladder tumor containing four signals for both centromere 9 and centromere 1,  $\times 630$

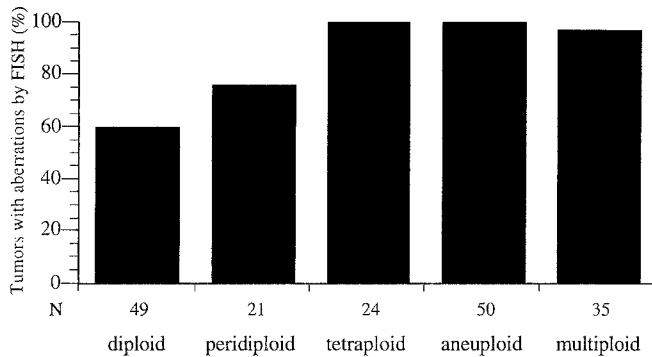


**Fig. 2** Comparison of percentage of cells with polysomy 17 found in two separate hybridizations of 173 tumors. Each circle represents one tumor.  $r^2 = 0.908$ ,  $n = 173$ ,  $P < 0.0001$

## Results

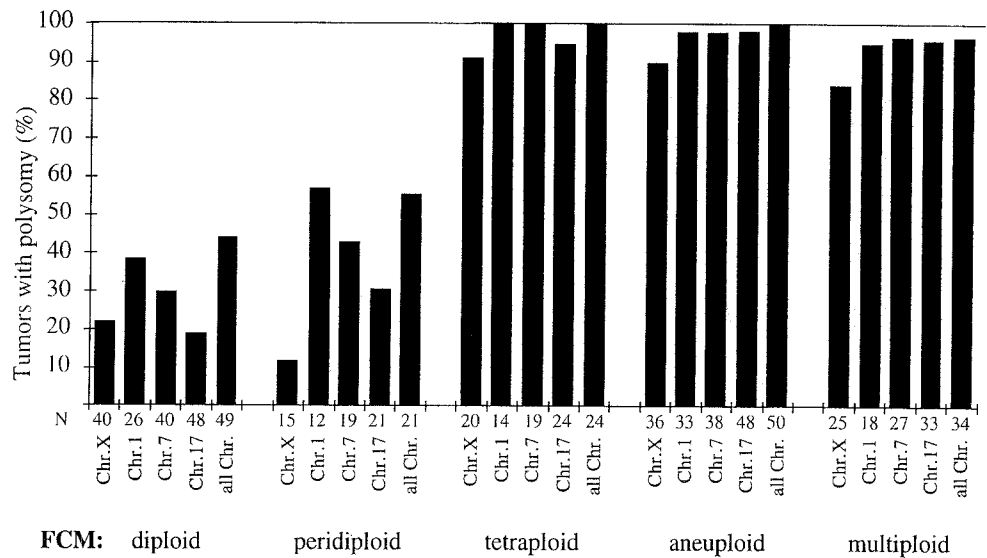
### Flow cytometry

The mean coefficient of variation (CV) was 6.2 (range 2.8–18.1) in all 237 tumors analyzed by FCM. Ploidy was determined in the 193 (81%) cases with a diploid CV  $\leq 8\%$ . Fifty-two of these tumors were diploid, 21 were peridi-



**Fig. 3** FISH aberrations in tumors with differing ploidy type. Bars indicate the frequency in which polysomies or losses of at least one chromosome (X, Y, 1, 7, 9, 17) occurred in tumors with differing ploidy types

**Fig. 4** Polysomies in tumors with differing ploidy type. The frequency of polysomies of chromosomes X, 1, 7, and 17 are shown as separate bars for tumors with differing ploidy types



**Table 1** Predominant populations in bladder tumors examined by FISH

FCM:	diploid	peridiploid	tetraploid	aneuploid	multiploid			
Chromosome	Sex	N	Predominant population (tumors %)					
			0	1	2	3	4	>4
Chromosome Y		125	<u>29</u>	<b>34</b>	34	1	2	0
Chromosome X	M	131	0	<b>45</b>	51	3	1	0
	F	53	0	<u>15</u>	<b>26</b>	26	23	10
Chromosome 1		127	0	<u>2</u>	<b>27</b>	33	33	5
Chromosome 7		181	0	<u>0.5</u>	<b>32.5</b>	22	38	7
Chromosome 9		186	0	<u>18</u>	<b>40</b>	19	20	3
Chromosome 17		213	0	<u>0.5</u>	<b>40</b>	20.5	34	5

<sup>a</sup> Largest aneusomic population having either  $\geq 10\%$  polysomic cells or  $\geq 20\%$  cells with less than the expected number of chromosomes

<sup>b</sup> The expected number of chromosomes is printed in bold for each chromosome. Tumors with chromosomal losses are underlined

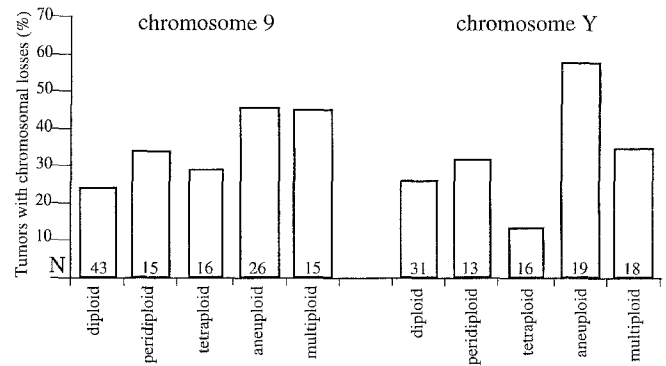
ploid, 25 were tetraploid, 55 were aneuploid, and 40 were multiploid.

### FISH analysis

FISH analyses with centromeric probes were successful in most cases. The slides were interpretable for centromere X in 184 of 191 (96%), for centromere Y in 125 of 133 (94%), for chromosome 1 in 127 of 135 (95%), for chromosome 7 in 181 of 199 (91%), for chromosome 9 in 186 of 215 (87%), and for chromosome 17 in 238 of 280 tumors (85%). Fifteen tumors were examined for one, 30 for two, 47 of three, 39 for four, 66 for five, and 56 for all six chromosomes. Most tumors were heterogeneous, containing more than one significant population with identical centromere counts. A cell of a representative case hybridized with probes for the centromeres 1 and 9 is shown in Fig. 1. The fraction of polysomic cells ranged between 0% and 100% for all chromosomes. A second hybridization for chromosome 17 was performed in 173 tumors. This showed that the fraction of polysomic cells was highly reproducible (Fig. 2). A polysomy ( $>2$  centromere signals in  $\geq 10\%$  of cells) was found for chromosome X in 58%, for chromosome Y in 40%, for chromosome 1 in 76%, for chromosome 7 in 68%, for chromosome 9 in 52%, and for chro-

**Table 2** Diploid tumors with single aberrations (NE not examined). Abnormal populations are shown in bold

Tumor	Sex	Histology	FCM	n chr. <sup>a</sup>	Aberration	Chr. X		Chr. Y		Chr. 1		Chr. 7		Chr. 9		Chr. 17	
						Loss <sup>b</sup>	Poly. <sup>c</sup>	Loss <sup>b</sup>	Poly. <sup>c</sup>	Loss <sup>b</sup>	Poly. <sup>c</sup>	Loss <sup>b</sup>	Poly. <sup>c</sup>	Loss <sup>b</sup>	Poly. <sup>c</sup>	Loss <sup>b</sup>	Poly. <sup>c</sup>
2356	M	pTa/G1	Diploid	6	Loss Y	0	0	<b>89</b>	0	2	0	0	0	3	0	4	0
12169	M	pTa/G1	Diploid	6	Loss 9	0	3	0	3	7	7	6	5	<b>92</b>	0	6	3
14140	M	pTa/G1	Diploid	5	Loss Y	4	5	<b>95</b>	0	1	1	NE	NE	11	0	7	5
23557	M	pTa/G1	Diploid	5	Loss Y	0	0	<b>98</b>	0	NE	NE	3	0	2	0	8	1
35472	M	pTa/G1	Diploid	6	Polysomy 1	0	0	7	7	2	<b>10</b>	0	0	9	2	5	1
11546	M	pTa/G2	Diploid	5	Polysomy 7	1	1	3	1	NE	NE	0	<b>82</b>	7	1	7	0
14708	M	pTa/G2	Diploid	5	Polysomy 7	2	0	3	0	NE	NE	5	<b>14</b>	6	0	8	1
15476	M	pTa/G2	Diploid	6	Polysomy 1	0	2	2	9	2	<b>14</b>	3	3	7	4	9	1
15315	F	pTa/G3	Diploid	4	Polysomy 1	NE	NE	NE	NE	2	<b>87</b>	3	4	8	3	6	1
12581	F	pT2/G2	Diploid	5	Polysomy X	2	<b>11</b>	NE	NE	4	1	1	1	8	0	5	1

<sup>a</sup> Number of chromosomes examined<sup>b</sup> Percentage of cells with fewer than the expected number of signals<sup>c</sup> Percentage of polysomic cells (cells with more than the expected number of signals)**Fig. 5** Chromosomal losses and ploidy. The frequency of chromosome 9 and Y losses in tumors with different FCM findings is depicted as *separate bars*

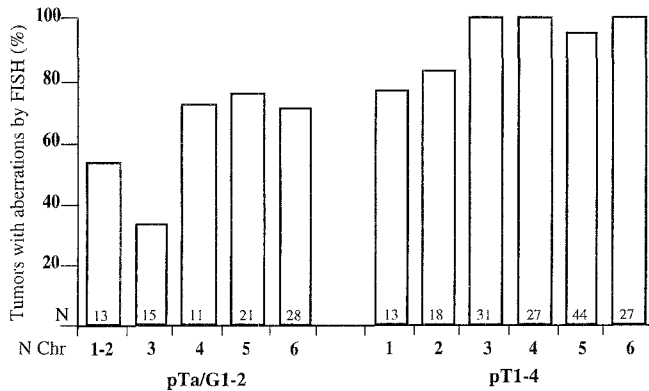
mosome 17 in 64%. A polysomy of at least one chromosome was seen in 191 of 273 tumors (75.5%) examined by FISH. The distribution of the predominant populations for all chromosomes in all tumors is shown in Table 1. Fewer than the expected number of chromosomes were mostly found for chromosome Y (29% of tumors), and chromosome 9 (18% of tumors). Interestingly there was a monosomy X in 15% of the examined female patients while a nullisomy X was never observed in male patients. Chromosomal losses were rare ( $\leq 2\%$ ) for the chromosomes 1, 7, and 17. A chromosome 9 loss according to our definition (including monosomy 9 and relative chromosome 9 losses) was found in 36% of tumors.

### FISH and FCM

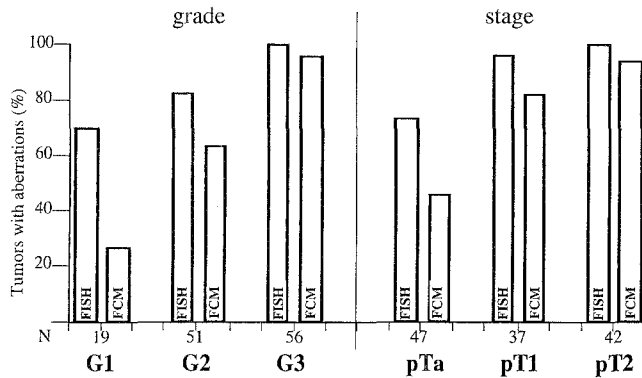
Chromosomal aberrations by FISH were found in 107 of 108 tumors that were tetraploid, aneuploid, or multiploid by FCM (Fig. 3). One multiploid tumor with a total of 37% aneuploid cells by FCM showed no chromosomal aberrations by FISH. However, only two chromosomes had been examined in this case. Numerical aberrations of at least one chromosome were also detected in 16 of 21 peridiploid (76%), and 29 of 49 diploid tumors (59%). A separate analysis of polysomies and chromosomal losses showed that polysomies were strongly associated with presence of tetraploid/aneuploid/multiploidy populations by FCM ( $P < 0.001$ ; Fig. 4) while there was no significant relationship between losses of the chromosomes Y and 9 and FCM ploidy (Fig. 5).

### DNA aberrations and tumor phenotype

As a rule the fraction of tumors showing chromosomal aberrations increased with the number of FISH probes used (Fig. 6). This was prominent when one to four FISH probes were applied while there was no further increase in the number of polysomies detected when four to six probes were employed. Presence of a major DNA aberration by FCM (tetraploidy, aneuploidy, or multiploidy) and poly-



**Fig. 6** Relationship between the frequency of aneusomy detection and the number of FISH probes applied. The percentage of tumors with detected polysomy or loss of at least one of the chromosomes X, Y, 1, 7, 9, and 17 as related to the number of chromosomes examined is shown separately for pTa/G1–2 tumors (*left*) and invasive carcinomas (pT1–4). *N* number of tumors examined, *N Chr* number of chromosomes examined



**Fig. 7** Relationship of FISH and FCM findings with tumor grade and stage. The frequency of detected aberrations is shown as bars for each grade and stage. Tumors with less than four chromosomes examined by FISH were excluded from this analysis

somies as detected by FISH were strongly associated with a high tumor grade and an invasive tumor stage (pT1–4). The fraction of detectable aberrations increased continuously from grade 1 over grade 2 to grade 3 tumors with highly significant differences between grade 1 and grade 2 tumors ( $P < 0.0001$ ) and between grade 2 and grade 3 tumors ( $P < 0.0001$ , Fig. 7). A marked difference was also seen between pTa and pT1 carcinomas ( $P < 0.0001$ ), while there was no significant further increase in the number of aberrations from pT1 to pT2–4 carcinomas. In contrast to polysomies, losses of the chromosomes Y and 9 were not significantly associated with tumor grade and stage (data not shown).

## Discussion

These results show that alterations of the cellular DNA content occur frequently in bladder carcinomas. This is

consistent with a number of previous studies using image cytometry, flow cytometry, or FISH for detection of DNA aberrations [1, 8, 9, 18, 23]. Importantly, the differences in aneuploidy and polysomies of all chromosomes examined were clearly more prominent between pTa and pT1 tumors than between pT1 and pT2–4 tumors. The concept that pT1 tumors are biologically closer to pT2–T4 tumors is also supported by marked differences in p53 lesions and other cytogenetic alterations between pTa and pT1 carcinomas [19, 20]. These results argue against the current concept of grouping pTa and pT1 tumors together as “superficial bladder carcinomas.”

Three of the FISH probes used in this study were selected because alterations of these chromosomes are known to occur frequently in bladder tumors. Chromosome 9 carries one or several tumor suppressor genes with relevance for bladder cancer, loss of chromosome Y has been suggested to have prognostic significance in bladder tumors, and for chromosome 7 it was suggested that a trisomy could represent an initial event in bladder carcinogenesis [11, 16, 17]. The chromosomes X, 1, and 17 were selected because high-quality centromere probes were available.

Our FISH analyses of multiple chromosomes in individual tumors showed that polysomies were usually not restricted to one chromosome but involved several if not all chromosomes examined. This suggests that most chromosomal aberrations which are detectable by FISH are non-specific, being the result of an event involving more or less the entire genome. This fits well with models suggesting that progression of malignant tumors goes along with sequential changes of their cellular DNA content. It is believed that early genomic alteration includes losses or gains of a few chromosomes or subchromosomal regions that can result in a peridiploid DNA histogram [4]. Development of aneuploidy is likely to occur through tetraploidization of previously diploid or peridiploid tumors and a subsequent loss of chromosomal material [23]. Our hypothesis that most polysomies detected by FISH may be the result of a previous tetraploidization is further supported by the equally high frequencies of polysomies of all chromosomes examined in tumors with an FCM result consistent with a previous tetraploidization (tetraploid, aneuploid, and multiploid tumors) as shown in Fig. 4.

To evaluate whether numerical aberrations of the chromosomes examined can be specific events potentially involved in the initiation of bladder cancer, we screened for tumors having only one chromosomal aberration. Such tumors are likely to be diploid by FCM, since the sensitivity of FCM is not sufficient to allow detection of gains or losses of single chromosomes in formalin-fixed tissues. Among 126 tumors which were examined for at least 4 chromosomes by FISH and also by FCM, there were only 10 diploid tumors having a numerical alteration of a single chromosome (Table 2). While it cannot be excluded that these ten tumors had aberrations of other chromosomes not examined in this study, this result shows that sole numerical aberrations of the selected chromosomes are rare in bladder cancer. Five different aberrations were found

among ten tumors having only one aberration and no single chromosome was solely involved in more than three tumors. Therefore, an important role of numerical aberration of the examined chromosomes for bladder cancer initiation seems unlikely. This does not rule out, however, that an altered function of genes on these chromosomes – for example MTS1/2 on chromosome 9 – is important for bladder cancer development.

The comparison of FISH and FCM findings revealed a superior sensitivity of FISH for detection of DNA aberrations. The finding of chromosomal aberrations by FISH in all but one of 108 tumors found to be tetraploid, aneuploid, or multiploid by FCM is comparable to the results of previous studies on prostate, bladder, and ovarian carcinomas, where the fraction of FCM aneuploid tumors without detectable aberrations by FISH was low [7]. The finding of chromosomal aberrations in 59% of the FCM diploid tumors is also in agreement with a previous study comparing FISH and FCM in bladder cancer [7]. Interestingly the fraction of tumors with aberrations was only slightly higher in peridiploid than in diploid tumors. It is possible that the application of additional probes could have resulted in a better result in the subset of peridiploid tumors, even though the application of more than four probes did not further improve the sensitivity of FISH in the entire patient set.

There was no association between chromosome Y and 9 losses and FCM aneuploidy in this set of patients. This observation is consistent with two different types of “genetic instability” in bladder cancer. One type of instability obviously allows tetraploidization and development of aneuploidy. An inactivation of the p53 tumor suppressor gene is likely to be involved in the pathway leading to this type of instability since p53 alterations are strongly linked to polysomies in bladder cancer [19, 21, 22]. It is estimated that mutations or deletions of p53 occur in more than 50% of invasive bladder tumors [2, 3, 13, 15, 27]. The conspicuous lack of an association between chromosomal losses and FCM aneuploidy raises the possibility that the development of minor chromosomal aberrations including chromosome Y or 9 losses is facilitated by a different type of “genetic instability.” Such a second mechanism could be independent of p53 alterations since losses of the chromosomes Y and 9 were not related to p53 immunostaining in previous studies [21, 22].

A major problem in the management of bladder cancer patients is the diagnosis of recurrent bladder tumors. Although urinary cells are readily accessible for cytological examination, the sensitivity (and specificity) of cytology alone is poor in low-grade tumors [12]. Previous studies have suggested that DNA measurement of urinary cells by FCM or image cytometry could be a valuable tool for the diagnosis of bladder cancer recurrences (reviewed in [26]). The results of this study indicate that the sensitivity of FISH for detection of aberrant urinary cells could be even higher than the sensitivity of FCM. Importantly the sensitivity of FISH is particularly high in grade 1 tumors, where the sensitivity of urinary cytology ranges between 16% and 30%. In this study DNA aberrations were detected by FCM

in 26% and by FISH in 68% of grade 1 tumors. Considering that not all FISH probes applied in this study were selected for chromosomes or loci known to be frequently affected in low-grade bladder tumors, it appears likely that FISH detection of aberrant urinary cells could be further improved by an optimized set of probes. If so, FISH could be a powerful tool for urinary tract cytology, even though its specificity will not reach 100% because of rare chromosomal aberrations in normal urothelium [22].

In summary, these data show that an aberrant DNA content is frequent in bladder carcinomas. The striking difference in DNA aberrations between pTa and pT1 tumors challenges the concept of grouping these tumors together as “superficial bladder carcinomas.” The high sensitivity of FISH for detection of DNA alterations in grade 1 tumors suggests that the cytological diagnosis of bladder cancer recurrences could be substantially improved by FISH. This is especially true because FISH is a simple method requiring very little material with the potential for routine application.

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